

AWARD NUMBER: W81XWH-15-1-0425

TITLE: Identifying Neurofibromin-Specific Regulatory Nodes for Therapeutic Targeting in NF1

PRINCIPAL INVESTIGATOR: Frank P. McCormick

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, CA 94103

REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 2016		2. REPORT TYPE Annual		3. DATES COVERED 30 Sep 2015 -29 Sept 2016	
4. TITLE AND SUBTITLE Identifying Neurofibromin-Specific Regulatory Nodes for Therapeutic Targeting in NF1				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-15-1-0425	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Frank McCormick, Evan Markegard, Osamu Tetsu, Ellen Mercado E-Mail: frank.mccormick@ucsf.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Helen Diller Family Comprehensive Cancer Center, University of California San Francisco, 94143				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The overall goal of the project is to determine how neurofibromin is regulated, and to explore the hypothesis that loss of neurofibromin activity leads to up-regulation of specific receptors. We are building on our earlier discovery, that neurofibromin depends on the adapter protein SPRED1, to function, and we are utilizing the latest technical innovations including CRISPR technology to find genes that regulate neurofibromin SPRED function. To date we have demonstrated that oncogenic EGFR signaling disrupts Spred1-NF1 binding. Mass spectrometry was performed on cells overexpressing EGFR ^{L858R} to identify potential phosphorylation sites on Spred1 and NF1 that could disrupt Spred1-NF1 binding by steric hindrance. A serine phosphorylation site on Spred1 was identified in which a phosphomimetic and phosphodeficient mutant decreased or increased Spred1-NF1 binding, respectively. Therefore, phosphorylation of Spred1 at this site by a serine kinase downstream of oncogenic EGFR may disrupt Spred1-NF1 binding. Our findings provide one potential mechanism by which oncogenic EGFR signaling disrupts negative feedback to allow for constitutive Ras signaling. Furthermore, this work may elucidate a novel kinase therapeutic target for restoring NF1 mediated inhibition of Ras.					
15. SUBJECT TERMS Neurofibromin, Spred1, Spred2, neurofibromatosis, therapeutic targeting					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 21	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

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Introduction

The overall goal of the project is to determine how neurofibromin is regulated, and to explore the hypothesis that loss of neurofibromin activity leads to up-regulation of specific receptors. We hope that we will be able to find news of up-regulating neurofibromin in cells in which one allele is defective, and that we will be able to suppress hyperactive Ras signaling in cells in which both alleles are lost. We are building on our earlier discovery, that neurofibromin depends on the adapter protein SPRED1, to function, and we are utilizing the latest technical innovations including CRISPR technology to find genes that regulate neurofibromin SPRED function.

Keywords

Neurofibromin, Spred1, Spred2, EGFR, mutant EGFR(L858R), Ras-GTP, Ras-MAPK, RTK, neurofibromatosis, RasGAPs, growth factor signaling, therapeutic targeting.

Major Goals of the Project

Major Task 1: Determine and verify RTKs that interact with Spred1 and Spred2

Major Task 2: Determine effect of NF1/Spred loss on Spred-binding RTK signaling

Major Task 3: Determine EGFR-mediated changes in Spred and Neurofibromin phosphorylation and interaction partners

Major Task 4: Determine targets for increasing Spred/Neurofibromin interaction and NF1 function in NF1^{-/+} cells

Major Task 5: Determine novel post-translational modifications and interacting proteins with Neurofibromin

Accomplishments

Major Task 1: Determine and verify RTKs that interact with Spred1 and Spred2

We have generated many RTK plasmids including EGFR, EGFR(L858R), cMET, CKIT, CKIT(D816V), ErbB2, ErbB2(V654E), ErbB3, PDGFR1, FGFR1, FGFR2, CSF1R, FLT3, PDGFRA, PDGFRA(D842V), and PDGFRB. We have investigated the affinity for Spred1 to bind to mutant EGFR(L858R) over wild-type EGFR (Figure 1). EGFR inhibition decreases Spred1-EGFR(L858R) binding and EGF stimulation of wild-type EGFR decreases Spred1-EGFR binding. These results are surprising and important for understanding the role of Spred1 on both wild-type and pathogenic RTKs. We plan to follow up with Spred1 and Spred2 binding to other mutant and wild-type RTKs.

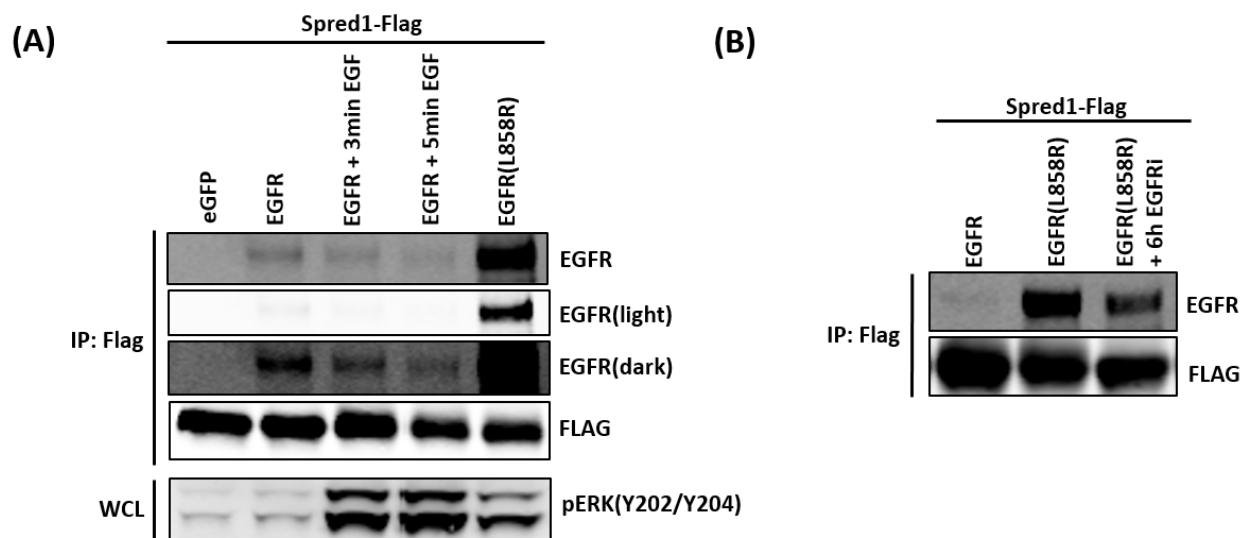


Figure 1. Spred 1 binds mutant EGFR(L858R) with higher affinity than wild-type EGFR. A) HEK 293T cells were transfected, serum starved for 16 hours, and stimulated with 20ng/ml EGF and EGFR binding accessed by Western blot. (B) Cells were transfected and Spred-NF1 bindings was assessed by Western blot. EGFR inhibitor AZD9291 used at 1 μ M for 6 hours.

Major Task 2: Determine effect of NF1/Spred loss on Spred-binding RTK signaling

NF1-Null HEK 293T cells have been generated using CRISPR/Cas9 and single clones have been expanded for biochemical assays (Figure 2). NF1-Null MEFs have also been generated and will be characterized. Spred1-Null HEK 293T and MEFs are currently being generated using CRISPR/Cas9.

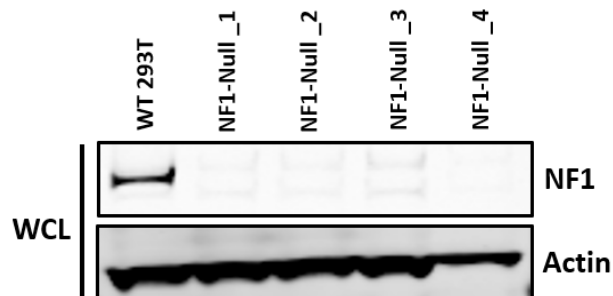


Figure 2. Clonal NF1-Null HEK 293T cells lines were generated using CRISPR/Cas9 with a sgRNA targeting exon 2 of NF1. Following antibiotic selection clones were screened by Western blot.

Ras-MAPK signaling in response to EGF has been analyzed in NF1 wild-type and null 293T cells (Figure 3). Both the magnitude and duration of Ras-MAPK signaling seems to be enhanced in the NF1-Null 293T cells compared to wild-type. Also, Spred1 overexpression is unable to inhibit Ras-MAPK signaling in NF1-null cells compared to wild-type.

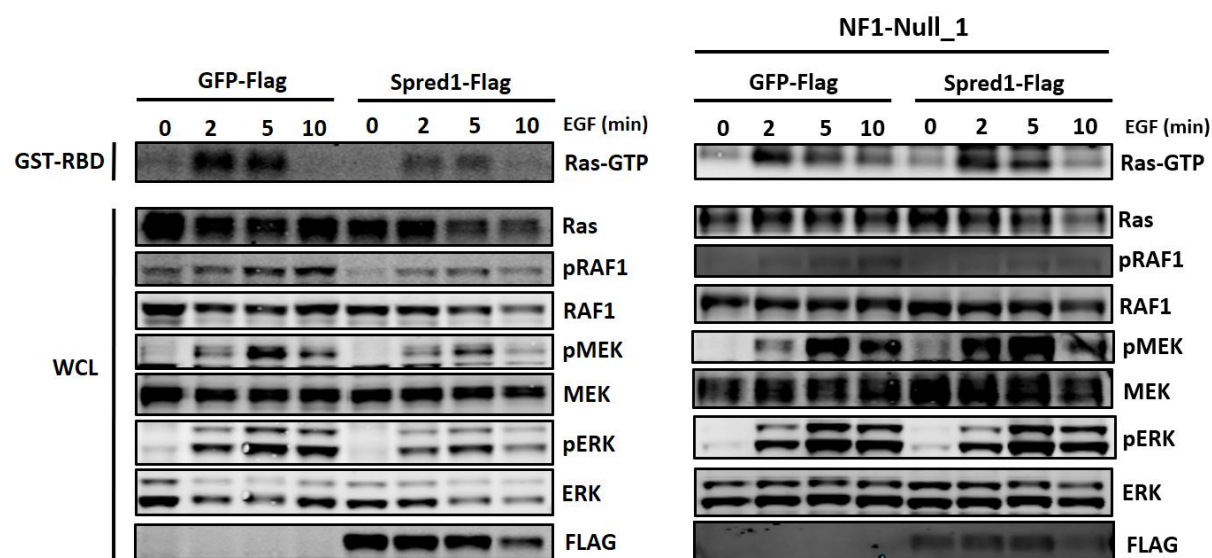


Figure 3. Spred1 overexpression is unable to suppress RasGTP following EGF stimulation in NF1-Null cells. NF1-Null_1 cells were transfected, serum starved for 16 hours, and stimulated with 20ng/ml EGF. Downstream signaling was accessed by Western blot

Major Task 3: Determine EGFR-mediated changes in Spred and Neurofibromin phosphorylation and interaction partners

Flag tagged Spred1 was overexpressed in 293T cells with and without EGFR(L858R). Spred1 was immunoprecipitated and subject to mass spectrometry analysis. Phosphorylation and binding partners with mutant EGFR are shown below in Figure 4.

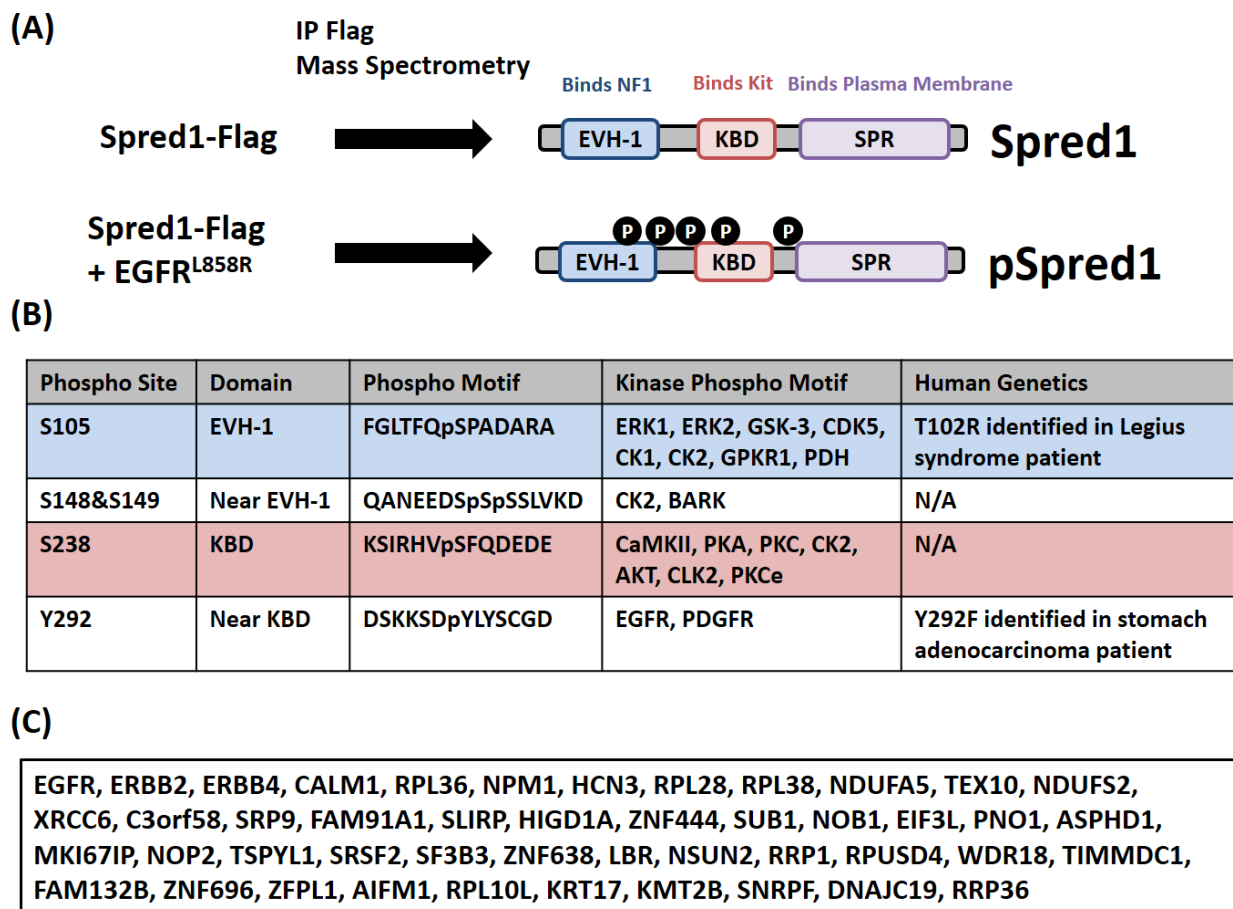


Figure 4. Identification of Spred1 phosphorylation and binding partners in the presence of mutant EGFR (L8585R). (A) Schematic for Spred1 mass spectrometry analysis. (B) Identification of Spred1 phosphorylation sites downstream of mutant EGFR. Kinase phosphorylation motifs identified using PhosphoMotif Finder and PhosphoSitePlus. (C) Spred1 interacting proteins identified by mass spectrometry.

Phosphodeficient and phosphomimic mutants of Spred1 phosphorylation sites identified by mass spectrometry were generated and tested for effects on NF1 binding. Spred1(S105) mutants showed the expected binding, shown below in Figure 5. Importantly, phosphomimic Spred1(S105D) is unable to bind NF1 and suppress Ras-GTP following EGF stimulation.

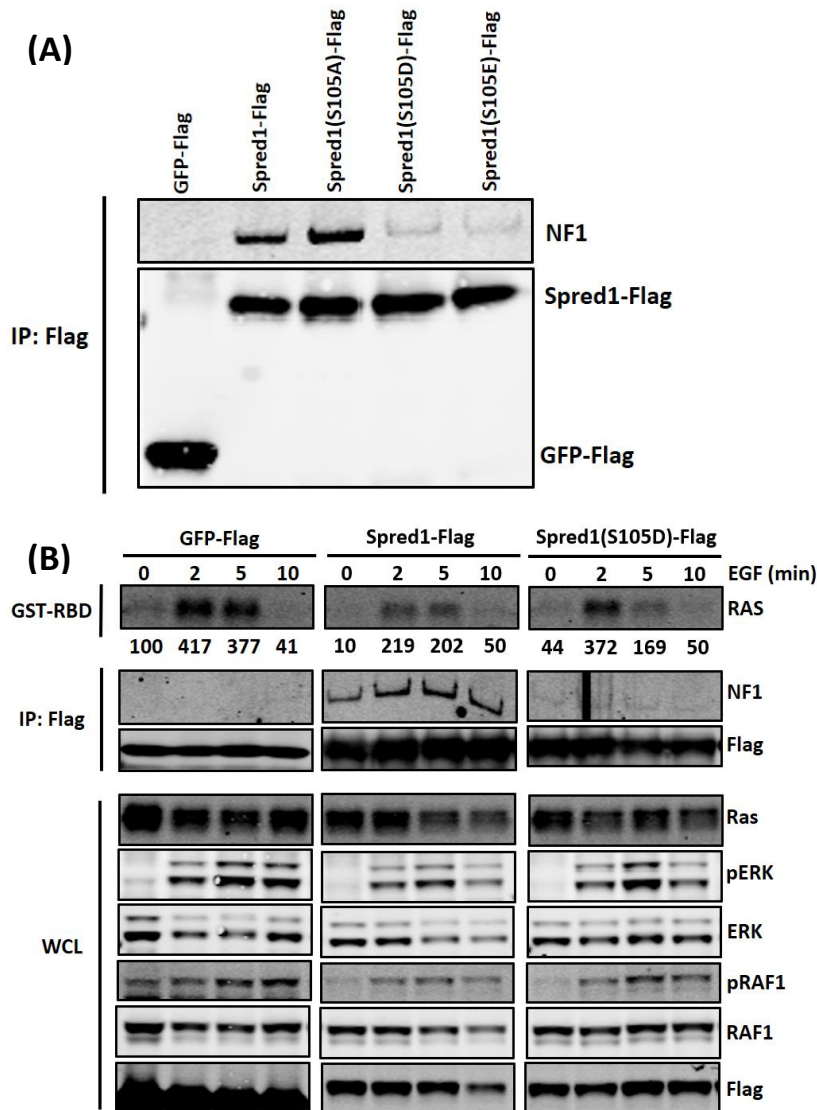


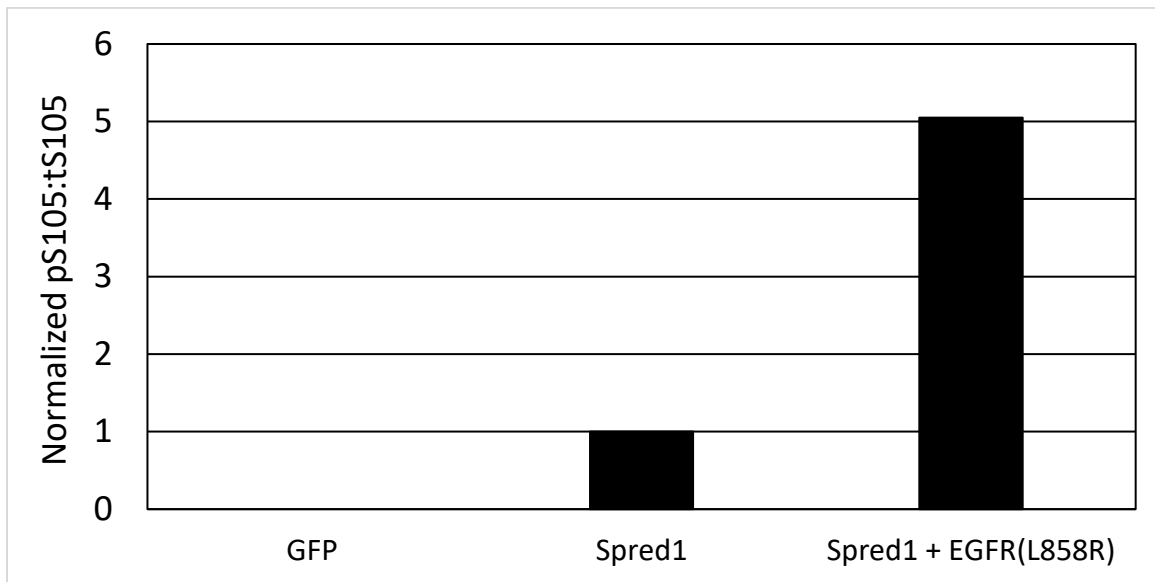
Figure 5. Phosphomimetic Spred1(S105) is unable to bind NF1 and suppress RasGTP following EGF stimulation. (A) Phosphomimetic and phosphodeficient Spred1 mutants at S105 were generated using site directed mutagenesis and transfected into HEK 293T cells to determine NF1 binding by Flag-IP followed by Western blot. (B) HEK 293T cells were transfected, serum starved for 16 hours, and stimulated with 20ng/ml EGF. Downstream signaling was accessed by Western blot.

To identify the Spred1(S105) kinase we are pursuing an in vitro kinase assay with Kinexus Bioinformatics, mass spectrometry experiments with candidate kinase inhibitors, and an unbiased CRISPRa screen. With Kinexus we are testing 93 candidate kinases with the Spred(S105) peptide based on the motif and EGFR pathway. These kinases are JNK1, JNK2, JNK3, P38 alpha, P38 beta, P38 delta, P38 gamma, GSK3 alpha, GSK3 beta, CK1 alpha 1, CK1 alpha 1L, CK1 delta, CK1 epsilon, CK1 gamma, 1 CK1 gamma, 2 CK1 gamma 3, CK2 alpha 1, CK2

alpha 2, CDK1/CyclinA1, CDK1/CyclinA2, CDK1/CyclinB1, CDK2/CyclinA1, CDK2/CyclinA2, CDK2/CyclinE1, CDK2/CyclinO, CDK3/CyclinE1, CDK4/CyclinD1, CDK4/CyclinD3, CDK5/p25, CDK5/p35, CDK6/Cyclin D1, CDK6/Cyclin D3, CDK7/CyclinH1/MNAT1, CDK9/CyclinK, CDK9/CyclinT2, (CDK16)/Cyclin Y PCTK2 (CDK17)/CyclinY PFTK1 (CDK14)/Cyclin Y, RAF1, BRAF, ERK1, ERK2, ERK5, MEK1, MEK2, MEK5, MEK6, AKT1, AKT2, AKT3, ALK1, ALK2, PKC alpha, PKC beta I, PKC beta II, PKC delta, PKC epsilon, PKC eta, PKC gamma, PKC iota, NLK, CHK1, CHK2, RSK1, RSK2, RSK3, RSK4, ICK, PCTK1, CAMKK2, SRPK2, COT, DYRK2, GRK1, PKC mu, PKC nu, PKC theta, PKC zeta, IKK alpha, IKK beta, IKK epsilon, CAMK1, CAMK1 beta, CAMK1 delta, CAMK1 gamma, CAMK2 alpha, CAMK2 beta, CAMK2, delta CAMK2, gamma CAMK4, CAMKK1, AMPK, CASK, MAK.

Phosphorylated Spred1(S105) has been quantitated and normalized to total Spred1(S105) using transient transfection in 293T cells and mass spectrometry as previously described. Expression of mutant EGFR(L858R) leads to a 5-fold increase in phospho-Spred1(S105). We will now test candidate kinase inhibitors which reduce phospho-Spred1(S105).

(A)



(B)

Candidate Kinase	Candidate Kinase Inhibitor
JNK	JNK-IN-8
CDK1/2/5	Dinaciclib
p38	LY2228820
GSK3beta	CHIR-99021
RAF1	LY3009120
PI3K	GDC-0941
PKC	Sotrastaurin

Figure 6. Design of mass spectrometry experiment to identify Spred1(S105) kinase. (A) Quantitation of relative phosphorylation at the S105 site in SPRED1 was carried out in Skyline v 3.0 by quantifying MS1 precursor peak areas of the S105-containing peptides and normalizing them by the sum of abundances of all unmodified peptides detected in the same protein. Transient transfection in HEK 293T was carried out as pervious described. (B) Candidate kinases and inhibitors to be tested to identify the Spred1(S105) kinase.

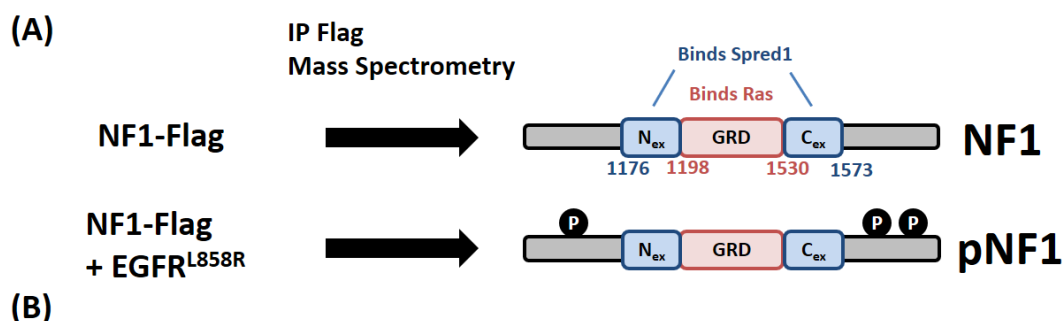
To identify genes that regulate NF1/SPRED function, including the Spred1(S105) kinase, we will perform an unbiased CRISPRa screen to activate genes in K562. To ensure hits are specific to NF1/Spred1 function we will counter screen in an isogenic K562 cell line in which we have knocked out NF1 using CRISPR. Recent CRISPRa screening data from our collaborator Dr. Michael Boettcher demonstrated overexpression of NF1, Spred1, and Spred2 all increase K562 sensitivity to imatinib, an approved BCR-ABL inhibitor. Given the robustness of these results, we believe K562 is an ideal cancer cell line system to interrogate regulators of NF1/SPRED function which can then be expanded to other disease models, as described below (“Changes” section).

Major task 4: Determine targets for increasing Spred/Neurofibromin interaction and NF1-/+ cells

When we have identified the kinase that regulates Spred/Neurofibromin interaction (above) we will test whether blocking this kinase promotes binding, as expected, and whether increased binding reduces Ras.GTP levels and prevents cells from growing. In addition, we will analyze genes that emerge from our functional CRISPR screen that are candidates for enzymes regulating Spred/neurofibromin interaction.

Major task 5: Determine novel post-translational modifications and interacting proteins with Neurofibromin

We have performed mass spectrometry on NF1 as described above with Spred1 to identify phosphorylation sites and binding partners important for function.



(B)

Phospho Site	Domain	Phospho Motif	Kinase Phospho Motif
S864	N/A	SGLATypSPPMGPV	GRK1, GSK-3, ERK1, ERK2, CDK5
S2597	N/A	PHLRKVpSVSESNV	CaMKII, PKA, PKC, PKCe, CK2, GSK-3, Phosphorylase, MAPKAPK2, GRK1
S2802	N/A	KENVELpSPTTGHC	GSK-3, ERK1, ERK2, CDK5, CK1, CK2

(C)

NONO, RSP16, HNRNPH2, TUBAL3, RPS8, C22orf28, SMARCA4, EEF1A1, EEF1A1P5, ATP5B, TUBB3, TUBB6, HSPA4, RPL6, NDUFA13, EEF1B2, LEPRE1, HSPA4L, HSPH1, RPS5, ZADH2, NDUFA4, DDX53, DHRS7B, RCN2, SMARCA2, DYNC1H1, MRPS27, DDX3Y, XRN2, DSP, CALM1, KHSRP, HNRNPF, RPLPO, RPLPOP6, ATAD3C, ATAD3A, CAD, FKBP8, PPM1B, COX20, HERC2P3, DDX6, KHDRBS1, ALDH3A2, RPS19, NPM1, FAM98A, FXR1,

Figure 7. Identification of NF1 phosphorylation and binding partners in the presence of mutant EGFR (L8585R). (A) Schematic for NF1 mass spectrometry analysis. (B) Identification of NF1 phosphorylation sites downstream of mutant EGFR. Kinase phosphorylation motifs identified using PhosphoMotif Finder. (C) NF1 interacting proteins identified by mass spectrometry.

We recently published our findings of the Spred1-binding domain of NF1, which flank the Gap Related Domain (GRD) domain (N_{ex} and C_{ex}) “The neurofibromin recruitment factor Spred1 binds to the GAP related domain without affecting Ras inactivation”, Theresia Dunzendorfer-Matt, Ellen L. Mercado, Karl Maly, Frank McCormick, and Klaus Scheffzek, *PNAS*, 113, 7497-7502, 2016.

Opportunities for training and professional Development

Graduate student Evan Markegard (funded by the project) is given one-on-one mentorship by Dr. McCormick which includes at least bi-weekly meetings either face-to-face or by skype to discuss his progress and goals. Evan presents his research at lab group meetings approximately four times per year, as well as at the UCSF Helen Diller Comprehensive Cancer Center “Research in Progress” seminar series and he presents at the Cancer Center annual retreat. This year he also presented a research poster at the American Association of Cancer Research 107th Annual Meeting in New Orleans in April this year (April 16-20, 2016). He will have the opportunity to present at a National or International Conference in each year of his graduate studies.

Dissemination of Results

- 1) Evan Markegard - Poster Presentation at the 107th Annual meeting of American Association of Cancer Research (April 16-20, 2016).

Abstract 1874: **Oncogenic EGFR signaling inhibits the Spred1-NF1 interaction to sustain constitutive Ras signaling.**

Evan Markegard, Ellen L. Mercado, Jacqueline Galeas, Marena I. Trinidad, Anatoly Urisman and Frank McCormick. **DOI:** 10.1158/1538-7445.AM2016-1874 Published 15 July 2016

- 2) Frank McCormick - Helen Diller Comprehensive Cancer Center Seminar Series (February 12, 2016)
- 3) Presentations and discussions at joint lab meetings with the labs of Ophir Klein (Department of Orofacial Sciences, UCSF), Natalia Jura (Department of Cellular and Molecular Pharmacology UCSF) and Kevin Shannon (Department of Pediatrics, UCSF).
- 4) Frank McCormick – Presentation at CTF Meeting, Austin TX June 2016

Impact

Nothing to report

Changes/Problems

While the Aims of the project remain unchanged, we will use new technologies to address these Aims. To identify proteins that regulate Neurofibromin and/or Spred, we will use functional screens in place of biochemical, mass spectrometry-based approaches. The latter approach has generated lists of proteins that appear to bind to Neurofibromin when the protein is expressed in cells in culture, but validation of these is challenging. We have been working closely with our UCSF colleagues Drs. Luke Gilbert, Michael Boettcher and Michael McManus to use CRISPRa and CRISPRi systems to identify regulatory proteins (Gilbert et al, *CELL*, 159, 647-661, 2014). We will use biochemical approaches to validate these hits, but strongly feel the primary screen should be functional. As described above, our colleagues performed a full genomic screen for genes that affect how K562 cells depend on imatinib. These cells were used because they grow in suspension, making full genome screens possible at high coverage. One of the strongest hits from the screen was Neurofibromin: over expression of this gene by CRISPRa mediated activation of the promoter, sensitized cells to the drug, whereas suppression made cells drug resistant. KRAS had exactly the opposite effect, as predicted. SPRED genes also score strongly in this system, behaving exactly as Neurofibromin (over-expression caused sensitivity, deletion caused resistance). In addition, we found that loss of Neurofibromin negated the toxic effects of SPRED over-expression, confirming that SPRED proteins (both SPRED1 and SPRED2) act through Neurofibromin. These results show that this system is ideal for screening for genes or drugs that regulate Neurofibromin or SPRED, as the cells are extremely sensitive to the activity of this pathway, and retain wild type, functional genes for the entire Ras pathway downstream of RTK signaling. We therefore expect this system to allow us to obtain candidate regulators, and will use biochemical screens in physiologically relevant cell systems to follow up on these hits, as described in the original proposal. An example of the power of this technology is shown in Figure 8.

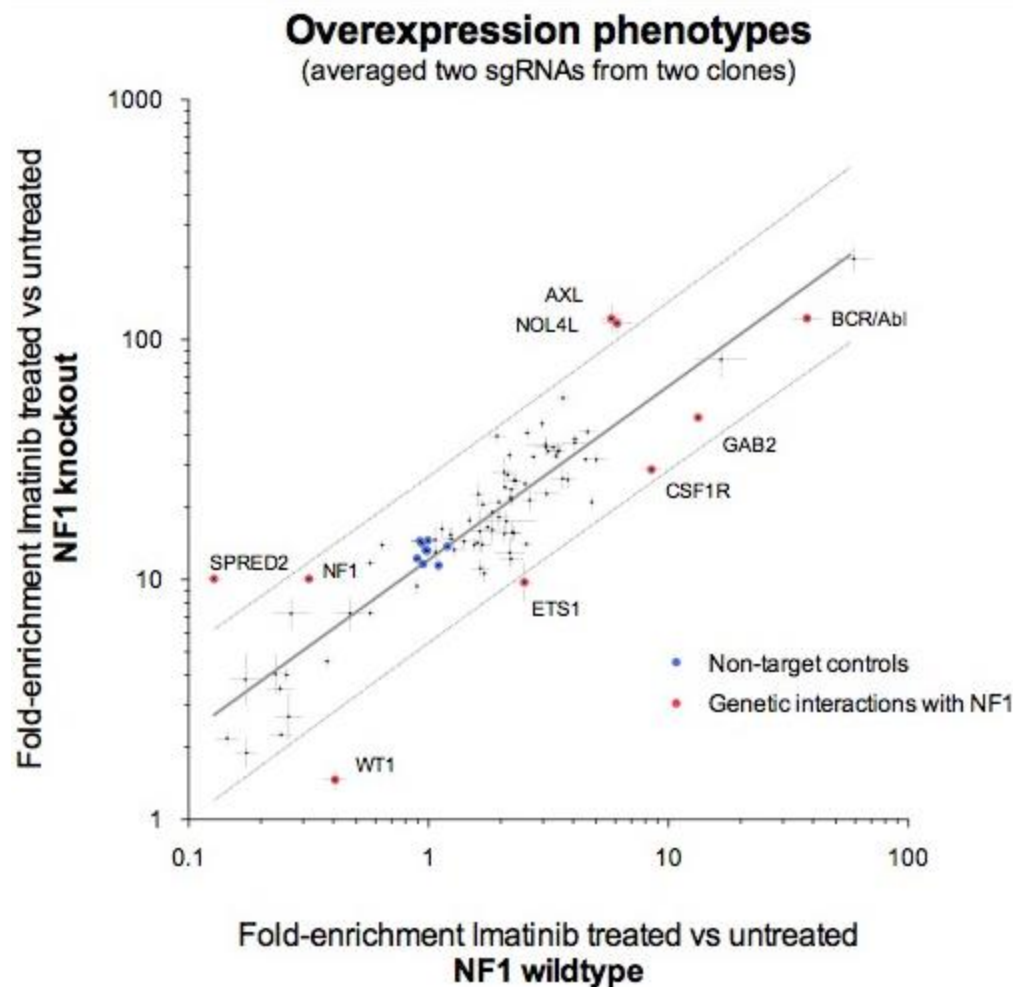


Figure 8. Screen for genes that differentially affect NF knockout cells vs wild type cells. A small set of genes were over-expressed using CRISPRa technology, in either background, and viability measured by deep sequencing.

In this screen, over-expression of SPRED2 or NF1 had no effect on viability of NF1 deficient cells (score of 10 represents no effect in NF1 KO cells), but both reduced viability of wild type cells (score of 1 represents no effect in wild type cells), as expected. However, over-expression of the receptor tyrosine kinase AXL and the AML fusion gene partner NOL4L greatly increased viability in the NF1 knockout cells, suggesting that these genes normally suppress growth through interaction with neurofibromin. Our efforts to identify tyrosine kinase receptors that are regulated by neurofibromin (described above) will now be expanded to include AXL. We will also use these preliminary data as the basis of a full genome wide screen to identify regulators of Neurofibromin and Spred signaling.

Products

Poster Presentation at the 107th Annual meeting of American Association of Cancer Research (April 16-20, 2016).

Abstract 1874: **Oncogenic EGFR signaling inhibits the Spred1-NF1 interaction to sustain constitutive Ras signaling.** Evan Markegard, Ellen L. Mercado, Jacqueline Galeas, Marena I. Trinidad, Anatoly Urisman and Frank McCormick. **DOI:** 10.1158/1538-7445.AM2016-1874 Published 15 July 2016

Participants and other collaborating organizations

We are collaborating with scientists at the Frederick National Labs to solve the structure of Neurofibromin, alone or in complexes with RAS and/or Spred proteins. Full length Neurofibromin made in baculovirus vectors has been extensively purified and shown to be an obligate dimer, using size-exclusion chromatography, sedimentation analysis and electron microscopy, as shown in Figure 9.

Neurofibromin is a dimer

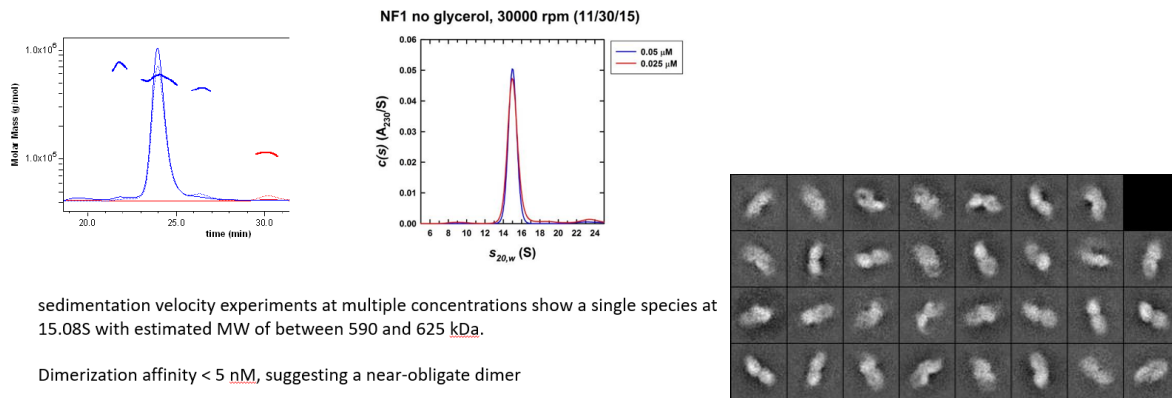


Figure 9. Biophysical characterization of recombinant, full length neurofibromin. Left panel: size exclusion chromatography. Middle panel, sedimentation analysis. Right panel, Negative-stain electron microscopy.

Individuals working on the project at UCSF

Name:	Evan Markegard
Project Role:	Graduate Student
Researcher Identifier:	
Nearest Person Month Worked:	12
Contribution to Project:	Evan performed many of the experiments in major tasks 1-4.
Funding Support:	This award

Name:	Frank McCormick
Project Role:	PI
Researcher Identifier:	
Nearest Person Month Worked:	1 (0.24)
Contribution to Project:	Oversight of all research
Funding Support:	This award

Name:	Osamu Tetsu
Project Role:	Senior investigator
Researcher Identifier:	
Nearest Person Month Worked:	2 (2.2)
Contribution to Project:	Dr. Tetsu investigated RAS activation via NF1 loss in EGFR mutant cancer cells and acted as an invaluable mentor for the postdoc and graduate student.
Funding Support:	This award

Name:	Ellen Mercado
Project Role:	Post Doc
Researcher Identifier:	
Nearest Person Month Worked:	4 months
Contribution to Project:	Dr. Mercado carried out the mass spectrometry analysis in major tasks 3 and 5.
Funding Support:	McCormick UCSF discretionary funds

Changes in PI support since Award Made:

Please see below for the Dr. Frank McCormick's (PI) active and pending other support:

ACTIVE**Funding Number:** 1R35CA197709

PI: McCormick, Frank

Project Title: *New Ways of Targeting K-Ras***Performance Period:** 04/01/2016-03/31/2023**Time Commitment:** 6.00 calendar months**Supporting Agency:** NIH/NCI**Contracting Officer:** Joanna Watson PhD., watsonjo@mail.nih.gov, (240) 276-6230**Level of Funding:** \$600,000 direct/yr 01**Project Goals:** To identify ways of suppressing the function of hyper-active Ras proteins for cancer therapy.**Specific Aims:** i) Analysis of GTPase Activating Proteins (GAPs) that regulate Ras; ii) analysis of distinct biochemical functions of different K-Ras mutants; iii) analysis of a novel, unique function of K-Ras 4B.**Overlap:** None**Funding Number:** U01 CA168370PI: McManus, Michael; McCormick, Frank;
Weissman, Jonathan**Project Title:** Bay Area Cancer Target Discovery and Development Network**Performance Period:** 05/01/12-04/30/2017**Time Commitment:** 0.24 calendar months**Supporting Agency:** NIH/NCI**Contracting Officer:** Daniela Gerhard PhD., gerharddd@mail.nih.gov, (301) 451-8027**Level of Funding:** \$99,228 direct/yr 04**Project Goals:** To bridge the gap between the enormous volumes of data generated by the comprehensive molecular characterization of a number of cancer types and the ability to use these data for the development of human cancer therapeutics. Our end goal is to generate game-changing reagents, and data valuable for the development of cancer therapeutics

Specific Aims: i) Develop next generation EXPAND libraries targeting cancer specific genetic alterations; ii) Identify recurrently mutated genes that regulate oncogenic pathways and drug responses; iii) Produce genetic interaction maps to uncover pathway relationships between candidate drivers.

Overlap: None

Funding Number: N/A

PI: McCormick, Frank

Project Title: Preclinical Evaluation of Signaling Pathways Involved in Cancer Malignancy

Performance Period: 03/22/2015 – 03/21/2017

Time Commitment: 0.12 calendar

Supporting Agency: Daiichi-Sankyo Company, Limited

Contracting Officer: Masashi Aonuma, PhD, VP, Oncology Research Laboratories,
aonuma.masahsi.jt@daiichisankyo.co.jp, +81-3-5696-8777

Level of Funding: \$189,274 direct/yr 02

Project Goals: Analysis of the Ras pathway in epithelial and mesenchymal cells.

Specific Aims: i) Determine how EMT affects MAPK and P13K pathways downstream of oncogenic Ras; ii) identify genes required for survival in epithelial and mesenchymal cells.

Overlap: None

Funding Number: N/A

PI: McCormick, Frank

Project Title: New ways of Treating Pancreatic Cancer Based on Reversing K-Ras-Mediated Stemness

Performance Period: 01/01/2015 – 12/31/2017

Time Commitment: 1.2 calendar months

Supporting Agency: Lustgarten Foundation

Contracting Officer: Christine Ardito-Abraham PhD., carditoa@lustgarten.org, (516) 803-2588

Level of Funding: \$410,447 direct/yr 02

Project Goals: Preclinical analysis of humanized anti-LIF monoclonal antibodies, using several models of pancreatic cancer, with the intention of moving these antibodies into clinical testing based on the results of these experiments

Specific Aims: i) generate human and mouse pancreatic cancer cells with LIF stably knocked down and elucidate the effects of this knock down; ii) validate LIF as a target of intervention for mouse pancreatic cancer in a syngenic mouse model; iii) compare the functions of LIF and IL6 in pancreatic cancer cells; iv) evaluate the therapeutic effects of LIF neutralizing antibody; v) elucidate the signaling pathways specifically driven by LIF and not other IL6 cytokine family members in K-Ras driven pancreatic cancers; vi) pre-clinically evaluate prostratin pro-drug in mouse models of pancreatic cancer; vii) investigate the mechanism of prostratin's tumor suppressing action on pancreatic cancer cell lines.

Overlap: None

Funding Number: N/A

PI: McCormick, Frank

Project Title: Targeting KRAS Mutant Lung Cancers

Performance Period: 08/01/2015—07/31/2018

Time Commitment: 1.2 calendar months

Supporting Agency: Subcontract through Massachusetts General Hospital

Contracting Officer: Maida Broudo, mbroudo@partners.org, (617) 816-7789

Level of Funding: \$384,750 direct/yr 02

Project Goals: Preclinical evaluation of prostratin and LIF for treating lung adenocarcinoma.

Specific Aims: i) Test the specific therapeutic effects of prostratin or its analogs on K-Ras driven lung cancers; 2) Examine the expression patterns of LIF in tumors and stromal cells.

Overlap: None

PENDING

None

Special Reporting Requirements - None

Appendices - None